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(54) Title: METHOD OF PROMOTING MYOCYTE PROLIFERATION AND MYOCARDIAL TISSUE REPAIR

(57) Abstract: The present invention provides methods, pharmaceutical compositions, improved cell culture medium and kits for promoting myocyte proliferation and myocardial tissue repair following myocardial injury by contact with angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and/or AII $AT_2$ type 2 receptor agonists.

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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | SUN YAO ET AL: "Angiotensin II, transforming growth factor-beta1 and repair in the infarcted heart." JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY, vol. 30, no. 8, August 1998 (1998-08), pages 1559-1569, XP002150870 ISSN: 0022-2828 the whole document | 1-35 |
| A | US 5 716 935 A (RODGERS K.E. ET AL.) 10 February 1998 (1998-02-10) the whole document | 1-35 |
| A | WO 98 26795 A (THE UNIVERSITY OF SOUTH CALIFORNIA) 25 June 1998 (1998-06-25) page 42 -page 44 | 1-35 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | SUN YAO ET AL: "Inhibition of angiotensin-converting enzyme and attenuation of myocardial fibrosis by lisinopril in rats receiving angiotensin II."<br>JOURNAL OF LABORATORY AND CLINICAL MEDICINE,<br>vol. 126, no. 1, 1995, pages 95-101,<br>XP000953010<br>ISSN: 0022-2143<br>the whole document | 1-35 |

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Information on patent family members

Int tional Application No
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(54) Title: METHOD OF PROMOTING MYOCYTE PROLIFERATION AND MYOCARDIAL TISSUE REPAIR

(57) Abstract

The present invention provides methods, pharmaceutical compositions, improved cell culture medium and kits for promoting myocyte proliferation and myocardial tissue repair following myocardial injury by contact with angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and/or AII $AT_2$ type 2 receptor agonists.

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# METHOD OF PROMOTING MYOCYTE PROLIFERATION AND MYOCARDIAL TISSUE REPAIR

## Cross Reference

This application claims priority to U.S. Provisional Patent Applications Serial Nos. 60/123,678 (filed March 9, 1999) and 60/151,874 filed August 31, 1999, both incorporated by reference herein in their entirety.

## Field of the Invention

This present invention relates to myocyte proliferation and differentiation and to myocardial tissue repair.

## Background of the Invention

Techniques for harvesting and culturing myocardial cells from a range of species, including adult human atrial myocardiocytes, have been established. (Smith et al., *In Vitro Cell. Dev. Biol.* 27A:914-920 (1991)). However, cardiac myocytes proliferate slowly if at all in culture (Kardami, *Mol. and Cell. Biochem.* 92:129-135 (1990)). The ability to manipulate these cells in vitro is extremely important for identifying growth factors pertinent to regeneration of heart cells after myocardial infarction or other ischemic injury.

Ventricular myocytes of the adult mammalian myocardium have traditionally been considered to be terminally differentiated cells, incapable of proliferation. (Kardami, *Mol. and Cell. Biochem.* 92:129-135 (1990)). Soon after birth these cells stop dividing and subsequent muscle growth is brought about by increases in cell size (hypertrophy) rather than cell number. *Id.* However, evidence indicates that ventricular myocytes have not lost their proliferative potential irreversibly, since they can be induced to synthesize DNA in culture. (Claycomb and Bradshaw, *Dev. Biol.* 90:331-337 (1983)). Atrial myocytes of the adult heart retain mitotic potential to a significant extent (Rumyanchev, *Int. Rev. Cytol.* 51:187-273 (1977)).

Techniques for the proliferation of human myocardial cells have been utilized, including the use of a platelet freeze-thaw extract (U.S. Patent Application 5,580,779, hereby incorporated by reference in its entirety). However, these methods are laborious, do not

utilize defined compounds for increasing myocyte proliferation, and provide limited increases in myocyte proliferation. Therefore, improved methods using defined compounds for inducing proliferation and differentiation of myocytes are needed.

Approximately 1.25 million Americans suffer from acute myocardial infarction (MI) each year, leading to more than 475,000 deaths per year. (Talbert, Am. J. Health Syst. Pharm. 54:S9-S16 (1997)). The cost of MI is estimated at more than $50 billion annually. Recent advances in the management of acute cardiac diseases, including acute MI, are resulting in an expanding patient population that will eventually develop chronic heart failure.

Early recognition and treatment of MI are important if outcomes are to be improved. Infarct size and location are key prognostic factors for outcomes after acute MI. (Talbert, 1997) Following MI, adverse ventricular remodeling by fibrous tissue occurs at the site of MI and remote to it. (Sun et al., Mol. Cell. Cardiol. 30:1559-1569 (1998)). It has been suggested that cardiac fibrosis plays a role in the development of congestive heart failure in post-MI heart. (Ju et al., Cardiovasc. Res. 35:223-232 (1997)). In the failing human heart of ischemic origin, fibrosis remote to the MI is considered the major feature of adverse tissue structure. Increased myocardial collagen concentration and abnormal matrix structure adversely alters myocardial stiffness, leading to ventricular diastolic dysfunction. (Sun et al., 1998). Damaged cardiac muscle is eventually replaced by scar tissue formed by non-muscle cells converging at the site of injury. This compromises cardiac performance further and shortens cardiac lifespan.

Reperfusion therapy is an accepted therapy for MI patients, and its application early in MI has been shown to reduce infarct size and increase survival. (Granger, Am. J. Cardiol. 79(12A):44-48 (1997); Talbert, 1997) A number of drug classes administered in this manner have been shown to result in smaller infarct size, including free radical scavengers, calcium antagonists, β blockers, magnesium, inhibitors of white blood cell function, inhibitors of cellular adhesion selectin molecules, adenosine (Granger, 1997), and fibroblast growth factor (U.S. Patent No. 4,296,100). Despite the benefits conferred by reperfusion therapy, there is evidence that such treatment can lead to "reperfusion injury", including microvascular damage and dysfunction. (Granger, 1997)

These methods have proven to be of limited efficacy in promoting repair of myocardial tissue after MI or for treating heart failure. Thus, there remains a need for the

development of methods to promote myocardial tissue repair following myocardial infarction that minimize fibrosis.

Recent studies have implicated angiotensin II (AII) and/or activation of the AII AT1 receptor in promoting fibrous tissue formation at MI and remote repair sites, while AII receptor antagonism has been shown in animal models to improve wound healing at MI and remote repair sites. (Sun et al., 1998; Ju et al., 1997; Sun, Adv. Exp. Med. Biol. 432:55-61 (1997); De Carvalho Frimm et al., Labor. and Clin. Med. 129:439-446 (1997); Thai et al., Am. J. Physiol. 276:H873-880 (1999); Tomita et al., Hypertension 32:273-279 (1998))

Current therapy for heart failure is primarily directed to using angiotensin-converting enzyme (ACE) inhibitors and diuretics (U.S. Patent No. 5,679,545). While prolonging survival in the setting of heart failure, ACE inhibitors appear to slow the progression towards end-stage heart failure, and substantial numbers of patients on ACE inhibitors have functional class III heart failure. Moreover, ACE inhibitors consistently appear unable to relieve symptoms in more than 60% of heart failure patients, and they reduce the mortality of heart failure only by approximately 15-20%. Heart transplantation is limited by the availability of donor hearts *Id.* Further, with the exception of digoxin, the chronic administration of positive inotropic agents has not resulted in a useful drug without accompanying adverse side effects, such as increased arrhythmogenesis, sudden death, or other deleterious side effects related to survival. These deficiencies in current therapy suggest the need for additional therapeutic approaches to heart failure.

The use of myocytes cultured *in vitro* as a therapeutic approach offers promise for the treatment of various cardiac disorders (U.S. Patent No. 5,679,545). As one specific example, myocytes may be implanted into a patient who has suffered a myocardial infarction prior to the onset of fibrosis, therefore potentially avoiding a weakening in the myocardium that may result in aneurysm formation. Alternatively, such myocytes may be used in aneurysm repair. In further embodiments, myocytes generated in culture may be used in conjunction with artificial materials to produce substrates for reconstructive cardiac surgery.

**Summary of the Invention**

In one aspect, the present invention provides methods, kits, and pharmaceutical compositions for increasing myocyte proliferation and differentiation by contacting the cells with angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof,

angiotensin II (AII), AII analogues, AII fragments or analogues thereof or AII $AT_2$ type 2 receptor agonists, either alone or in combination with other growth factors and cytokines.

The methods of this aspect of the invention may be used to treat heart failure, to provide myocardial cells that may be transplanted or implanted into a patient that suffers from a cardiac disorder, to study the physiology of cardiac muscle, or to identify pharmaceutical agents that may be useful in the treatment of heart disease.

In a further aspect, the present invention provides methods and kits to promote myocardial tissue repair following myocardial injury, comprising the administration of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments or analogues thereof or AII $AT_2$ type 2 receptor agonists to a patient in need thereof.

**Detailed Description of the Preferred Embodiments**

All references, patents and patent applications are hereby incorporated by reference in their entirety.

As defined herein, the term "myocyte" includes any myocardial cell, either fetal or adult in origin. Examples of myocytes include, but are not limited to, those described in U.S. Patent Application 5,580, 779; Smith et al., 1991 *supra*; and Kardami 1990, *supra*, all references hereby incorporated in their entirety. As defined herein, "proliferation" encompasses both cell self renewal and cellular proliferation with accompanying differentiation.

As defined herein, the term "myocardial injury" encompasses injuries including, but not limited to myocardial ischemia, myocardial infarction, cardiomyopathies, coronary artery disease, heart valve disease, myocarditis, and heart failure.

As used herein, the term "myocardial infarction" refers to adverse changes in the myocardium or heart muscle that results from obstruction of a coronary artery.

As used herein, the term "repair following myocardial infarction" refers to a decrease in the fibrosis and scarring that typically follows MI, and to promoting the production of healthy muscle and tissue at necrotic sites.

As used herein, the term "heart failure" refers to the failure of the heart to pump blood with normal efficiency and thus to provide adequate blood flow to other body organs. Heart failure may be due to failure of the right or left or both ventricles. The signs and symptoms of

heart failure depend upon which side of the heart is failing. They can include dyspnea, cardiac asthma, pooling of blood in the systemic circulation or in the liver's portal circulation, edema, cyanosis, and hypertrophy of the heart. There are many causes of congestive heart failure including but not limited to coronary artery disease leading to heart attacks and heart muscle weakness, primary heart muscle weakness from viral infections or toxins such as prolonged alcohol exposure, heart valve disease causing heart muscle weakness due to too much leaking of blood or heart muscle stiffness from a blocked valve, hypertension, hyperthyroidism, vitamin deficiencies, and drug use. The aim of therapy for heart failure is to improve the pumping function of the heart.

Unless otherwise indicated, the term "active agents" as used herein refers to the group of compounds comprising angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII) analogues, AII fragments or analogues thereof or AII $AT_2$ type 2 receptor agonists, either alone, combined, or in further combination with other compounds, for treating or preventing restenosis, such as anticoagulants, platelet aggregation inhibitors, smooth muscle cell proliferation inhibitors, calcium channel blockers, angiotensin converting enzyme inhibitors, angiotensin II receptor antagonists, and antilipidemics.

Unless otherwise indicated, the term "angiotensin converting enzyme inhibitors" or "ACE inhibitors" includes any compound that inhibits the conversion of the decapeptide angiotensin I to angiotensin II, and include but are not limited to alacepril, alatriopril, altiopril calcium, ancovenin, benazepril, benazepril hydrochloride, benazeprilat, benzazepril, benzoylcaptopril, captopril, captopril-cysteine, captopril-glutathione, ceranapril, ceranopril, ceronapril, cilazapril, cilazaprilat, converstatin, delapril, delapril-diacid, enalapril, enalaprilat, enalkiren, enapril, epicaptopril, foroxymithine, fosfenopril, fosenopril, fosenopril sodium, fosinopril, fosinopril sodium, fosinoprilat, fosinoprilic acid, glycopril, hemorphin-4, idapril, imidapril, indolapril, indolaprilat, libenzapril, lisinopril, lyciumin A, lyciumin B, mixanpril, moexipril, moexiprilat, moveltipril, muracein A, muracein B, muracein C, pentopril, perindopril, perindoprilat, pivalopril, pivopril, quinapril, quinapril hydrochloride, quinaprilat, ramipril, ramiprilat, spirapril, spirapril hydrochloride, spiraprilat, spiropril, spiropril hydrochloride, temocapril, temocapril hydrochloride, teprotide, trandolapril, trandolaprilat, utibapril, zabicipril, zabiciprilat, zofenopril and zofenoprilat. (See for example Jackson, et al., Renin and Angiotensin in Goodman & Gilman's The Pharmacological Basis of

Therapeutics, 9th ed., eds. Hardman, et al. (McGraw Hill, 1996); and U.S. Patent No. 5,977,159.)

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of healing of wound tissue, comprising the application to such tissue of angiotensin II (AII) in an amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen (*Circulation Research* 60:786-790 (1987); Clouston et al., *Genomics* 2:240-248 (1988); Kageyama et al., *Biochemistry* 23:3603-3609; Ohkubo et al., *Proc. Natl. Acad. Sci.* 80:2196-2200 (1983)); all references hereby incorporated in their entirety). The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the converting enzyme angiotensinase which removes the C-terminal His-Leu residues from AI, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu [SEQ ID NO:37]. AII is a known pressor agent and is commercially available.

Studies have shown that AII increases mitogenesis and chemotaxis in cultured cells that are involved in wound repair, and also increases their release of growth factors and extracellular matrices (diZerega, U.S. Patent No. 5,015,629; Dzau et. al., *J. Mol. Cell. Cardiol.* 21:S7 (Supp III) 1989; Berk et. al., *Hypertension* 13:305-14 (1989); Kawahara, et al., *BBRC* 150:52-9 (1988); Naftilan, et al., *J. Clin. Invest.* 83:1419-23 (1989); Taubman et al., *J. Biol. Chem.* 264:526-530 (1989); Nakahara, et al., *BBRC* 184:811-8 (1992); Stouffer

and Owens, *Circ. Res.* 70:820 (1992); Wolf, et al., *Am. J. Pathol.* 140:95-107 (1992); Bell and Madri, *Am. J. Pathol.* 137:7-12 (1990)). In addition, AII was shown to be angiogenic in rabbit corneal eye and chick chorioallantoic membrane models (Fernandez, et al., *J. Lab. Clin. Med.* 105:141 (1985); LeNoble, et al., Eur. J. Pharmacol. 195:305-6 (1991)).

We have previously demonstrated that angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments or analogues thereof; AII $AT_2$ type 2 receptor agonists (hereinafter referred to as the "active agents") are effective in accelerating wound healing and the proliferation of certain cell types. See, for example, co-pending U.S. Patent Application Serial Nos. 09/012,400 (January 23, 1998); 09/198,806 (November 24, 1998); 09/264,563 (Filed March 8, 1999); 09/287,674 (Filed April 7, 1999); 09/307,940(Filed May 10, 1999); 09/246,162 (Filed February 8, 1999); 09/255,136 (Filed February 19, 1999); 09/245,680 (Filed February 8, 1999); 09/250,703 (Filed February 15, 1999); 09/246,525 (Filed February 8, 1999); 09/266,293 (Filed March 11, 1999); 09/332,582 (Filed June 14, 1999); 09/373,962 (Filed August 13, 1999); and 09/352,191 (Filed March 11, 1999); as well as U.S. Patent Serial Nos. 5,015,629; 5,629,292; 5,716,935; 5,834,432; and 5,955,430; all references incorporated herein by reference in their entirety.

The effect of AII on a given cell type has been hypothesized to be dependent, in part, upon the AII receptor subtype(s) the cell expresses (Shanugam et al., *Am. J. Physiol.* 268:F922-F930 (1995); Helin et al., *Annals of Medicine* 29:23-29 (1997); Bedecs et al., *Biochem J.* 325:449-454 (1997)). These studies have shown that AII receptor subtype expression is a dynamic process that changes during development, at least in some cell types. AII activity is typically modulated by either or both the AT1 and AT2 AII receptors. However, AII has recently been shown to stimulate proliferation of primary human keratinocytes via a non-AT1, non-AT2 receptor. (Steckelings et al., Biochem. Biophys. Res. Commun. 229:329-333 (1996)). These results underscore the cell-type (ie: based on receptor expression) specific nature of AII activity.

The effects of AII receptor and AII receptor antagonists have been examined in two experimental models of vascular injury and repair which suggest that both AII receptor subtypes (AT1 and AT2) play a role in wound healing (Janiak et al., *Hypertension* 20:737-45 (1992); Prescott, et al., *Am. J. Pathol.* 139:1291-1296 (1991); Kauffman, et al., *Life Sci.*

49:223-228 (1991); Viswanathan, et al., *Peptides* 13:783-786 (1992); Kimura, et al., *BBRC* 187:1083-1090 (1992).

Many studies have focused upon AII(1-7) (AII residues 1-7) or other fragments of AII to evaluate their activity. AII(1-7) elicits some, but not the full range of effects elicited by AII. (Pfeilschifter, et al., *Eur. J. Pharmacol.* 225:57-62 (1992); Jaiswal, et al., *Hypertension* 19(Supp. II):II-49-II-55 (1992); Edwards and Stack, *J. Pharmacol. Exper. Ther.* 266:506-510 (1993); Jaiswal, et al., *J. Pharmacol. Exper. Ther.* 265:664-673 (1991); Jaiswal, et al., *Hypertension* 17:1115-1120 (1991); Portsi, et a., *Br. J. Pharmacol.* 111:652-654 (1994)).

Other data suggests that the AII fragment AII(1-7) acts through a receptor(s) that is distinct from the AT1 and AT2 receptors which modulate AII activity. (Ferrario et al., J. Am. Soc. Nephrol. 9:1716-1722 (1998); Iyer et al., Hypertension 31:699-705 (1998); Freeman et al., Hypertension 28:104 (1996); Ambuhl et al., Brain Res. Bull. 35:289 (1994)). Thus, AII(1-7) activity on a particular cell type cannot be predicted based solely on the effect of AII on the same cell type. In fact, there is some evidence that AII(1-7) often opposes the actions of AII. (See, for example, Ferrario et al., Hypertension 30:535-541 (1997))

Many studies have been conducted to assess the effect of AII on the cardiovascular system. Some studies have suggested that AII has a toxic effect on myocytes, by inducing cellular hypertrophy (in the absence of cell proliferation) in non-infarcted myocardium (Riegger, Cardiovasc. Drugs and Therapy 10:613-615 (1996); Kudoh et al., Circ. Res. 80:139-146 (1997); Hein et al., Proc. Natl. Acad. Sci. 94:6391-6396 (1997)). Other studies suggest that AII effects myocyte growth indirectly, via a fibroblast-derived factor that is increased by AII (Sil and Sen, Hypertension 30:209-216 (1997). Other studies suggest that the AT2 receptor can serve to mediate the antigrowth effects of AII (Booz and Baker, Hypertension 28:635-640, 1996). Thus, the effect of AII on myocyte proliferation is at best controversial, and the effects of angiotensinogen, AI, and AI and AII fragments and analogues is unknown.

Angiotensin II is also known as a potent stimulator of angiogenesis (Fernandez et al., *J. Lab. Clin. Med.* 105:141-145 (1985)), and has been shown to activate collateral circulation via preformed blood vessels in rat kidneys (Fernandez et al., *Am. J. Physiol.* 243:H869-H875 (1982)). However, recent studies have implicated angiotensin II and/or AII AT1 receptor activation in promoting fibrous tissue formation at MI and remote repair sites, and antagonism of the AII receptor has been shown in animal models to influence wound healing

at MI and remote repair sites. (Sun et al., 1998; Ju et al., 1997; Sun, Adv. Exp. Med. Biol. 432:55-61 (1997); De Carvalho Frimm et al., Labor. and Clin. Med. 129:439-446 (1997); Thai et al., Am. J. Physiol. 276:H873-880 (1999); Tomita et al., Hypertension 32:273-279 (1998)) Thus, the use of the active agents of the invention to promote myocardial tissue repair following myocardial infarction would be unexpected.

A peptide agonist selective for the AT2 receptor (AII has 100 times higher affinity for AT2 than AT1) is p-aminophenylalanine6-AII ["(p-$NH_2$-Phe)6-AII"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein Xaa is p-$NH_2$-Phe (Speth and Kim, *BBRC* 169:997-1006 (1990). This peptide gave binding characteristics comparable to AT2 antagonists in the experimental models tested (Catalioto, et al., *Eur. J. Pharmacol.* 256:93-97 (1994); Bryson, et al., *Eur. J. Pharmacol.* 225:119-127 (1992).

As hereinafter defined, a preferred class of AT2 agonists for use in accordance with the present invention comprises AII analogues or active fragments thereof having p-NH-Phe in a position corresponding to a position 6 of AII. In addition to peptide agents, various nonpeptidic agents (e.g., peptidomimetics) having the requisite AT2 agonist activity are further contemplated for use in accordance with the present invention.

The active AII analogues, fragments of AII and analogues thereof of particular interest in accordance with the present invention comprise a sequence consisting of at least three contiguous amino acids of groups $R^1$-$R^8$ in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which $R^1$ and $R^2$ together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is absent, H or a one to three peptide group,

$R^A$ is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, $Me^2$Gly, Pro, Bet, Glu($NH_2$), Gly, Asp($NH_2$) and Suc,

$R^B$ is suitably selected from Arg, Lys, Ala, Citron, Orn, Ser(Ac), Sar, D-Arg and D-Lys,

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Lys, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, Tyr($PO_3$)$_2$, Thr, Ser, homoSer, azaTyr, and Ala;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including $R^4$ as a terminal Tyr group.

Compounds falling within the category of AT2 agonists useful in the practice of the invention include the AII analogues set forth above subject to the restriction that $R^6$ is p-$NH_2$-Phe.

Particularly preferred combinations for $R^A$ and $R^B$ are Asp-Arg, Asp-Lys, Glu-Arg and Glu-Lys. Particularly preferred embodiments of this class include the following: AIII or AII(2-8), Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]; AII(3-8), also known as des1-AIII or AIV, Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:3]; AII(1-7), Asp-Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:4]; AII(2-7). Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:5]; AII(3-7), Val-Tyr-Ile-His-Pro [SEQ ID NO:6]; AII(5-8), Ile-His-Pro-Phe [SEQ ID NO:7]; AII(1-6), Asp-Arg-Val-Tyr-Ile-His [SEQ ID NO:8]; AII(1-5), Asp-Arg-Val-Tyr-Ile [SEQ ID NO:9]; AII(1-4), Asp-Arg-Val-Tyr [SEQ ID NO:10]; and AII(1-3), Asp-Arg-Val [SEQ ID NO:11]. Other preferred embodiments include: Arg-norLeu-Tyr-Ile-His-Pro-Phe [SEQ ID NO:12] and Arg-Val-Tyr-norLeu-His-Pro-Phe [SEQ ID NO:13]. Still another preferred embodiment encompassed within the scope of the invention is a peptide having the sequence Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe [SEQ ID NO:31]. AII(6-8), His-Pro-Phe [SEQ ID NO:14] and AII(4-8), Tyr-Ile-His-Pro-Phe [SEQ ID NO:15] were also tested and found not to be effective.

Another class of compounds of particular interest in accordance with the present invention are those of the general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which $R^2$ is selected from the group consisting of H, Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ser, homoSer, azaTyr, and Ala;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

A particularly preferred subclass of the compounds of general formula II has the formula

$R^2$-$R^3$-Tyr-$R^5$-His-Pro-Phe [SEQ ID NO:16]

wherein $R^2$, $R^3$ and $R^5$ are as previously defined. Particularly preferred is angiotensin III of the formula Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]. Other preferred compounds include peptides having the structures Arg-Val-Tyr-Gly-His-Pro-Phe [SEQ ID NO:17] and Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:18]. The fragment AII(4-8) was ineffective in repeated tests; this is believed to be due to the exposed tyrosine on the N-terminus.

In a preferred embodiment, the active agents comprise a sequence according to the general formula:

Asp-Arg-R1-Tyr-Ile-His-Pro-R2

wherein R1 is selected from the group consisting of Val, Pro, Ile, norLeu, Ile, Lys, Ala, Gly, Aib, Acpc and Tyr; and

wherein R2 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

Other particularly preferred embodiments include:

| | | | |
|---|---|---|---|
| 1GD | Ala4-AII(1-7) | DRVAIHP | SEQ ID NO:38 |
| 2GD | Pro3-AII(1-7) | DRPYIHP | SEQ ID NO:39 |
| 5GD | Lys3-AII(1-7) | DRKYIHP | SEQ ID NO:40 |
| 9GD | NorLeu-AII(1-7) | DR(nor)YIHP | SEQ ID NO:41 |
| GSD 28 | $Ile^8$-AII | DRVYIHPI | SEQ ID NO:42 |
| | Ala3aminoPhe6 AII: | DRAYIF*PF | SEQ ID NO:43 |
| | Ala3-AIII | RVAIHPF | SEQ ID NO:44 |
| | $Gly^1$-AII | GRVYIHPF | SEQ ID NO:45 |
| | $NorLeu^4$-AIII | --RVYnLHPF | SEQ ID NO:46 |
| | $Acpc^3$-AII | DR(Acpc)YIHPF | SEQ ID NO:47 |
| GSD 37B | $Orn^2$-AII | D(Orn)VYIHPF | SEQ ID NO:48 |
| GSD38B | $Citron^2$-AII | D(Citron)VYIHPF | SEQ ID NO:49 |
| 3GD | $Pro^3Ala^4$-AII(1-7) | DRPAIHP | SEQ ID NO:50 |

In the above formulas, the standard three-letter abbreviations for amino acid residues are employed. In the absence of an indication to the contrary, the L-form of the amino acid is intended. Other residues are abbreviated as follows:

TABLE 1

Abbreviation for Amino Acids

| | |
|---|---|
| $Me^2Gly$ | N,N-dimethylglycyl |
| Bet | 1-carboxy-N,N,N-trimethylmethanaminium hydroxide inner salt (betaine) |
| Suc | Succinyl |
| Phe(Br) | p-bromo-L-phenylalanyl |
| azaTyr | aza-α'-homo-L-tyrosyl |
| Acpc | 1-aminocyclopentane carboxylic acid |
| Aib | 2-aminoisobutyric acid |
| Sar | N-methylglycyl (sarcosine) |
| Cit | Citron |
| Orn | Ornithine |

It has been suggested that AII and its analogues adopt either a *gamma* or a *beta* turn (Regoli, et al., *Pharmacological Reviews* 26:69 (1974). In general, it is believed that neutral side chains in position $R^3$, $R^5$ and $R^7$ may be involved in maintaining the appropriate distance between active groups in positions $R^4$, $R^6$ and $R^8$ primarily responsible for binding to receptors and/or intrinsic activity. Hydrophobic side chains in positions $R^3$, $R^5$ and $R^8$ may also play an important role in the whole conformation of the peptide and/or contribute to the formation of a hypothetical hydrophobic pocket.

Appropriate side chains on the amino acid in position $R^2$ may contribute to affinity of the compounds for target receptors and/or play an important role in the conformation of the peptide. For this reason, Arg and Lys are particularly preferred as $R^2$. Alternatively, $R_2$ may be H, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg, or D-Lys.

For purposes of the present invention, it is believed that $R^3$ may be involved in the formation of linear or nonlinear hydrogen bonds with $R^5$ (in the gamma turn model) or $R^6$ (in the beta turn model). $R^3$ would also participate in the first turn in a beta antiparallel structure (which has also been proposed as a possible structure). In contrast to other positions in general formula I, it appears that beta and gamma branching are equally effective in this position. Moreover, a single hydrogen bond may be sufficient to maintain a relatively stable conformation. Accordingly, $R^3$ may suitably be selected from Lys, Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr.

With respect to $R^4$, conformational analyses have suggested that the side chain in this position (as well as in $R^3$ and $R^5$) contribute to a hydrophobic cluster believed to be essential for occupation and stimulation of receptors. Thus, $R^4$ is preferably selected from Tyr, Thr, Tyr $(PO_3)_2$, homoSer, Ser and azaTyr. In this position, Tyr is particularly preferred as it may form a hydrogen bond with the receptor site capable of accepting a hydrogen from the phenolic hydroxyl (Regoli, et al. (1974), *supra*). It has also been found that $R^4$ can be Ala.

In position $R^5$, an amino acid with a β aliphatic or alicyclic chain is particularly desirable. Therefore, while Gly is suitable in position $R^5$, it is preferred that the amino acid in this position be selected from Ile, Ala, Leu, norLeu, and Val.

In the active agents of particular interest in accordance with the present invention, $R^6$ is His, Arg or 6-$NH_2$-Phe. The unique properties of the imidazole ring of histidine (e.g., ionization at physiological pH, ability to act as proton donor or acceptor, aromatic character) are believed to contribute to its particular utility as $R^6$. For example, conformational models suggest that His may participate in hydrogen bond formation (in the *beta* model) or in the second turn of the antiparallel structure by influencing the orientation of $R^7$. Similarly, it is presently considered that $R^7$ should be Pro or Ala in order to provide the most desirable orientation of $R^8$. In position $R^8$, both a hydrophobic ring and an anionic carboxyl terminal appear to be particularly useful in binding of the analogues of interest to receptors; therefore, Tyr, Ile, Phe(Br), and especially Phe are preferred for purposes of the present invention.

Analogues of particular interest include the following:

TABLE 2

Angiotensin II Analogues

| AII Analogue Name | Amino Acid Sequence | Sequence Identifier |
|---|---|---|
| Analogue 1 | Asp-Arg-Val-Tyr-Val-His-Pro-Phe | SEQ ID NO: 19 |
| Analogue 2 | Asn-Arg-Val-Tyr-Val-His-Pro-Phe | SEQ ID NO: 20 |
| Analogue 3 | Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe | SEQ ID NO: 21 |
| Analogue 4 | Glu-Arg-Val-Tyr-Ile-His-Pro-Phe | SEQ ID NO: 22 |
| Analogue 5 | Asp-Lys-Val-Tyr-Ile-His-Pro-Phe | SEQ ID NO: 23 |
| Analogue 6 | Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe | SEQ ID NO: 24 |
| Analogue 7 | Asp-Arg-Val-Thr-Ile-His-Pro-Phe | SEQ ID NO: 25 |
| Analogue 8 | Asp-Arg-Val-Tyr-Leu-His-Pro-Phe | SEQ ID NO: 26 |
| Analogue 9 | Asp-Arg-Val-Tyr-Ile-Arg-Pro-Phe | SEQ ID NO: 27 |
| Analogue 10 | Asp-Arg-Val-Tyr-Ile-His-Ala-Phe | SEQ ID NO: 28 |
| Analogue 11 | Asp-Arg-Val-Tyr-Ile-His-Pro-Tyr | SEQ ID NO: 29 |
| Analogue 12 | Pro-Arg-Val-Tyr-Ile-His-Pro-Phe | SEQ ID NO: 30 |
| Analogue 13 | Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe | SEQ ID NO: 31 |
| Analogue 14 | Asp-Arg-Val-Tyr$(PO_3)_2$-Ile-His-Pro-Phe | SEQ ID NO: 32 |
| Analogue 15 | Asp-Arg-norLeu-Tyr-Ile-His-Pro-Phe | SEQ ID NO: 33 |
| Analogue 16 | Asp-Arg-Val-Tyr-norLeu-His-Pro-Phe | SEQ ID NO: 34 |
| Analogue 17 | Asp-Arg-Val-homoSer-Tyr-Ile-His-Pro-Phe | SEQ ID NO: 35 |

The polypeptides of the instant invention may be synthesized by any conventional method, including, but not limited to, those set forth in J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984) and J. Meienhofer, *Hormonal Proteins and Peptides*, Vol. 2, Academic Press, New York, (1973) for solid phase synthesis and E. Schroder and K. Lubke, *The Peptides*, Vol. 1, Academic Press, New York, (1965) for solution synthesis. The disclosures of the foregoing treatises are incorporated by reference herein.

In general, these methods involve the sequential addition of protected amino acids to a growing peptide chain (U.S. Patent No. 5,693,616, herein incorporated by reference in its entirety). Normally, either the amino or carboxyl group of the first amino acid and any reactive side chain group are protected. This protected amino acid is then either attached to an inert solid support, or utilized in solution, and the next amino acid in the sequence, also suitably protected, is added under conditions amenable to formation of the amide linkage. After all the desired amino acids have been linked in the proper sequence, protecting groups

and any solid support are removed to afford the crude polypeptide. The polypeptide is desalted and purified, preferably chromatographically, to yield the final product.

Preferably, peptides are synthesized according to standard solid-phase methodologies, such as may be performed on an Applied Biosystems Model 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.), according to manufacturer's instructions. Other methods of synthesizing peptides or peptidomimetics, either by solid phase methodologies or in liquid phase, are well known to those skilled in the art.

Alternatively, the peptides can be produced by standard molecular biological techniques.

In one aspect of the present invention, methods, kits, and pharmaceutical compositions for increasing *in vivo*, *in vitro* and *ex vivo* myocyte proliferation by exposure to angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII analogues, AII fragments or analogues thereof or AII $AT_2$ type 2 receptor agonists (hereinafter referred to as the "active agents") is disclosed. Experimental conditions for the isolation, purification, *in vitro*/*ex vivo* growth and *in vivo* mobilization of myocytes have been reported (Smith et al., 1991 *supra*; Kardami 1990, *supra*; U.S. Patent No. 5,580,779; Sil and Sen, Hypertension 30:209-216, 1997; Jacobsen et al., Basic Res. Cardiol. 1:79-82 (1985); Sen et al. J. Biol. Chem. 263:19132-19136 (1988)).

Proliferation can be quantitated using any one of a variety of techniques well known in the art, including, but not limited to, bromodeoxyuridine incorporation (Vicario-Abejon et al., 1995); Lazarous et al. Biotechnol. and Histochem. 67:253-255 (1992)), $^3$H-thymidine incorporation (Fredericksen et al., 1988), or antibody labeling of a protein present in higher concentration in proliferating cells than in non-proliferating cells. In a preferred embodiment, proliferation of myocytes is assessed by reactivity to an antibody directed against a protein known to be present in higher concentrations in proliferating cells than in non-proliferating cells, including but not limited to proliferating cell nuclear antigen (PCNA, or cyclin; Zymed Laboratories, South San Francisco, California).

Differentiation of myocytes is detected by immunohistochemistry using antibodies specific for muscle myosin, myoglobin, and atrial natriuretic peptide (ANP), as described in U.S. Patent No. 5,580,779.

In one embodiment, myocytes are isolated from atrial tissue according to standard methods (Smith et al., 1991, *supra*), suspended in culture medium, and incubated in the

presence of, preferably, between about 0.1 ng/ml and about 10 mg/ml of the active agents of the invention. The cells are expanded for a period of between 8 and 21 days and cellular proliferation is assessed as described above.

In a preferred embodiment, myocytes are isolated from atrial tissue obtained from cardiovascular surgery patients undergoing procedures requiring "heart-lung bypass." Smith et al., *In Vitro Cell Dev. Biol.* 27A:914-920 (1991). The samples are placed in an ice-saline slush immediately after removal, rinsed in saline, and the epicardial covering is removed with a scalpel to reduce the amount of connective tissue included in the cell harvest. The remaining "pure" atrial muscle is minced into 0.5 to 1.0 $mm^3$ pieces and placed in cold Hanks' balanced salt solution (HBSS) without calcium or magnesium (Whitaker; Walkerville, MD). The minced atrial tissue is digested in 0.14% collagenase solution (Worthington, Freehold, NJ) at a concentration of 1.43 mg/ml. The pieces are placed in 35 ml of this solution and digested in a shaker at 37°C at 125 rpm for one hour. The supernatant is removed from the atrial tissue and centrifuged at 3500 rpm for 10 minutes at 37°C. Another 35 ml of collagenase solution is placed on the minced tissue and the digestion is continued for another hour. Cell pellets are resuspended in 2 ml of Eagle's minimal essential medium (EMEM) with Earle's salts (Whitaker) containing 30% newborn bovine serum (Whitaker) and 0.1% antibiotic solution. Several digestions are conducted in this manner, and final cell concentrations are checked using a hemocytometer and adjusted to 1 x $10^5$ with EMEM, and then incubated with the active agents.

Myocytes exposed to the active agents as described above can be used, for example, for implantation or transplantation into a patient in need of such treatment. In this manner, autologous or heterologous cells may be implanted or transplanted into a patient who suffers from a cardiac disorder. As one specific example, cells may be implanted into a patient who has suffered a myocardial infarction prior to the onset of fibrosis, therefore potentially avoiding a weakening in the myocardium that may result in aneurysm formation (U.S. Patent No. 5,580,779). Alternatively, such cells or artificially produced myocardial tissue, may be used in aneurysm repair. In further embodiments, cells generated in culture may be used in conjunction with artificial materials to produce substrates for reconstructive cardiac surgery. In further specific embodiments, atrial myocardial cells caused to proliferate by the methods of the invention may be used in vivo or in vitro as a source of atrial natriuretic peptide. A cellular implant comprising such cells may be introduced into a patient as a source of atrial

natriuretic peptide that is subject to biofeedback mechanism. The cells are cultured *in vitro* or *ex vivo* as described above. The cells are rinsed to remove all traces of culture fluid, resuspended in an appropriate medium and then pelleted and rinsed several times. After the final rinse, the cells are resuspended at between 0.7 x $10^6$ and 50 x $10^6$ cells per ml in an appropriate medium and used as described.

In another aspect of the present invention the active agents are used to increase *in vivo* myocyte proliferation. In a preferred embodiment of this aspect, the active agent is administered by systemic infusion directly into the heart using an osmotic pump (Alza Palo Alto, CA) attached to a 30 gauge cannulae implanted at the injection coordinate, as described in Craig et al., J. of Neuroscience 16:2649-2658 (1996). In another preferred embodiment, the peptides are administered by infusion locally to the myocardium via an indwelling catheter.

A suitable injected dose of active agent is preferably between about 0.1 ng/kg and about 10 mg/kg administered twice daily. The active ingredient may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

In a further aspect, the present invention provides methods, pharmaceutical compositions, and kits to promote myocardial tissue repair after myocardial infarction, comprising administration of the active agents of the invention to a patient in need thereof to promote myocardial tissue repair after MI.

According to this aspect of the invention, an area of myocardial tissue is treated in vivo following, or at the time of, myocardial infarction (MI) to promote repair and lessened fibrosis. An effective dose of the active agents is applied to the myocardial tissue, preferably immediately following MI, although it can also be applied when there is an indication of impending MI.

In a preferred embodiment, a catheter is placed into the coronary artery of a subject between about at the time of to about 24 hours after myocardial infarction and injecting an effective amount of the active agent into the heart of the subject. The concentration of active agent injected is between about 100 ng/kg body weight and about 10.0 mg/kg body weight, as described above. The injection can be repeated as needed to promote myocardial tissue repair following MI. Injections can also be by other routes, including but not limited to by

catheter via arterial angiography, intracoronary injection, or in a cardioplegic solution by the aortic route.

For in vivo delivery, the active agents may be administered by any suitable route, including parentally, topically, or by cardiovascular devices in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intraarterial, intramuscular, intrasternal, intracardiac, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally. Furthermore, the active agents can be administered by gene therapy techniques.

The active agents of the invention may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The compounds of the invention may be applied in a variety of solutions. Suitable solutions for use in accordance with the invention are sterile, dissolve sufficient amounts of the peptide, and are not harmful for the proposed application. In this regard, the compounds of the present invention are very stable but are hydrolyzed by strong acids and bases. The compounds of the present invention are soluble in organic solvents and in aqueous solutions at pH 5-8.

The active agents may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as carriers, preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

The active agents of the invention can be used alone or in a combination of active agents, or may be used in combination with other agents that promote myocardial tissue repair, including, but not limited to free radical scavengers, calcium antagonists, β-blockers, magnesium, inhibitors of white blood cell function, inhibitors of cellular adhesion selectin molecules, adenosine, fibroblast growth factor, digoxin, and ACE inhibitors. Similarly, the active agents can be used in combination with other compounds that promote myocyte proliferation, differentiation, such as growth factors and cytokines including but not limited to epidermal growth factor, insulin-like growth factor, fibroblast growth factor, platelet derived growth factor, nerve growth factor, tumor necrosis factor, and interleukin I. When administered as a combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

For administration, the active agents are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

The dosage regimen for the therapeutic methods of the invention is based on a variety of factors, including the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Dosage levels of the order of between 0.1 ng/kg and 10 mg/kg body weight active agent per body weight are useful for all methods of use disclosed herein.

The treatment regime will also vary depending on the condition of the subject, based on a variety of factors, including the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. For example, an active agents is administered to a patient as soon as possible after, or at the time of, myocardial infarction and continuing for up to 30 days. The therapy is administered for 1 to 6 times per day at dosages as described above.

In a preferred embodiment, the active agent is administered via local delivery using cardiovascular devices. Local delivery of the active agents of the invention can be by a variety of techniques that administer the agent at or near the traumatized vascular site. Examples of site-specific or targeted local delivery techniques are not intended to be limiting but to be illustrative of the techniques available. Examples include local delivery catheters, such as an infusion catheter, an indwelling catheter, or a needle catheter, synthetic grafts, adventitial wraps, shunts and stents or other implantable devices, site specific carriers, direct

injection, or direct applications. (U.S. Patent 5,981,568, incorporated by reference herein in its entirety.)

Local delivery by an implant describes the surgical placement of a matrix that contains the active agent into the lesion or traumatized area. The implanted matrix can release the active agent by diffusion, chemical reaction, or solvent activators. See, for example, Lange, Science, 249, 1527 (1990).

An example of targeted local delivery by an implant is the use of a stent, which is designed to mechanically prevent the collapse and re-occlusion of the coronary arteries or other vessels. Incorporation of an active agent into the stent permits delivery of the active agent directly to the lesion. Local delivery of agents by this technique is described in Koh, Pharmaceutical Technology (October, 1990). For example, a metallic, plastic or biodegradable intravascular stent is employed which comprises the active agent. The stent may comprise a biodegradable coating, a porous or a permeable non-biodegradable coating, or a biodegradable or non-biodegradable membrane or synthetic graft sheath-like coating, e.g., PTFE, comprising the active agent. Alternatively, a biodegradable stent may also have the active agent impregnated therein, i.e., in the stent matrix.

A biodegradable stent with the active agent impregnated therein can be further coated with a biodegradable coating or with a porous non-biodegradable coating having a sustained release-dosage form of the active agent dispersed therein. This stent can provide a differential release rate of the active agent, i.e., there can be an initial faster release rate of the active agent from the coating, followed by delayed release of the active agent impregnated in the stent matrix, upon degradation of the stent matrix. The intravascular stent also provides a mechanical means of providing an increase in luminal area of a vessel.

Another example of targeted local delivery by an implant is the use of an adventitial wrap. The wrap comprises a pharmaceutically acceptable carrier matrix, including but not limited to a Pluronic gel which is free, or contained by a collagen mesh, which gel has dispersed therein the active agent.

Another embodiment of the invention is the incorporation of the active agent into the expanded nodal spaces of a PTFE (Impra, Inc., Tempe, Ariz.) vascular graft-like membrane which can surround, or be placed on the interior or on the exterior surface of, an interlumenal vascular stent, which comprises metal or a biodegradable or nonbiodegradable polymer. The

active agent, or a sustained release dosage form of the active agent, fills the nodal spaces of the PTFE membrane wall and/or coats the inner and/or outer surfaces of the membrane.

A suitable local delivery dose of active ingredient of active agent is preferably between about 0.1 ng/kg and about 10 mg/kg administered twice daily for a time sufficient to promote myocardial tissue repair following MI. In a more preferred embodiment, the concentration of active agent is between about 100 ng/kg body weight and about 10.0 mg/kg body weight. In a most preferred embodiment, the concentration of active agent is between about 10 μg/kg body weight and about 10.0 mg/kg body weight. This dosage regimen maximizes the therapeutic benefits of the subject invention while minimizing the amount of active agent needed. Such an application minimizes costs as well as possible deleterious side effects. when used in combination with existing therapies, the invention further minimizes the amount of other costly therapeutics, such as growth factors and cytokines.

In another preferred embodiment of the present invention, the active agent is administered parentally. Suitable topical doses and active ingredient concentration in the formulation are as described for local delivery via cardiovascular devices.

In a further preferred embodiment of all of the aspects of the invention, the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50.

In a further aspect, the present invention provides kits for promoting in vivo myocyte proliferation and differentiation, or myocardial tissue repair following MI, wherein the kits comprise an effective amount of active agent for promoting in vivo myocyte proliferation or myocardial tissue repair following MI, and instructions for using the amount effective of active agent as a therapeutic. In a preferred embodiment, the kit further comprises a pharmaceutically acceptable carrier, such as those adjuvants described above. In another

preferred embodiment, the kit further comprises a means for delivery of the active agent to a patient. Such devices include, but are not limited to infusion catheters, indwelling catheters, needle catheters, synthetic grafts, adventitial wraps, shunts, stents or other implantable devices, syringes, matrical or micellar solutions, bandages, wound dressings, aerosol sprays, lipid foams, transdermal patches, topical administrative agents, polyethylene glycol polymers, carboxymethyl cellulose preparations, crystalloid preparations (e.g., saline, Ringer's lactate solution, phosphate-buffered saline, etc.), viscoelastics, polyethylene glycols, and polypropylene glycols. The means for delivery may either contain the effective amount of active agent, or may be separate from the compounds, which are then applied to the means for delivery at the time of use.

In a further preferred embodiment, the kits further comprise an amount effective to promote in vivo myocyte proliferation and differentiation, or repair of myocardial tissue of at least one compound selected from the group consisting of free radical scavengers, calcium antagonists, β-blockers, magnesium, inhibitors of white blood cell function, inhibitors of cellular adhesion selectin molecules, adenosine, fibroblast growth factor, other growth factors, cytokines, digoxin, and ACE inhibitors.

In another aspect of the present invention, an improved cell culture medium is provided for the proliferation and differentiation of myocytes, wherein the improvement comprises addition to the cell culture medium of an effective amount of the active agents, as described above. Any cell culture media that can support the growth of myocytes can be used with the present invention. Such cell culture media include, but are not limited to Basal Media Eagle, Dulbecco's Modified Eagle Medium, Iscove's Modified Dulbecco's Medium, McCoy's Medium, Minimum Essential Medium, F-10 Nutrient Mixtures, Opti-MEM® Reduced-Serum Medium, RPMI Medium, and Macrophage-SFM Medium or combinations thereof.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as either a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement. Culture media is commercially available from many sources, such as GIBCO BRL (Gaithersburg, MD) and Sigma (St. Louis, MO)

In a further aspect, the present invention provides kits for the propagation of myocytes, wherein the kits comprise an effective amount of the active agents, as described

above, and instructions for using the active agents to promote myocyte proliferation and differentiation.

In a preferred embodiment, the kit further comprises cell culture growth medium. Any cell culture media that can support the growth and differentiation of myocytes can be used with the present invention. Examples of such cell culture media are described above.

The improved cell culture medium can be supplied in either a concentrated (ie: 10X) or non-concentrated form, and may be supplied as either a liquid, a powder, or a lyophilizate. The cell culture may be either chemically defined, or may contain a serum supplement.

In another preferred embodiment, the kit further comprises a sterile container. The sterile container can comprise either a sealed container, such as a cell culture flask, a roller bottle, or a centrifuge tube, or a non-sealed container, such as a cell culture plate or microtiter plate (Nunc; Naperville, IL).

In a further preferred embodiment, the kit further comprises an antibiotic supplement for inclusion in the reconstituted cell growth medium. Examples of appropriate antibiotic supplements include, but are not limited to actimonycin D, Fungizone®, kanamycin, neomycin, nystatin, penicillin, streptomycin, or combinations thereof (GIBCO).

The present invention, by providing a method for enhanced proliferation of myocytes, will greatly increase the clinical benefits of myocyte cell therapy after various ischemic events, including but not limited to myocardial infarction. This is true both for increased "self-renewal" of myocytes, which will provide a larger supply of myocytes at the appropriate site. Similarly, methods that increase *in vivo* proliferation of myocytes are beneficial in treating various ischemic events, including but not limited to myocardial infarction, aneurysm repair, and reconstructive cardiac surgery.

The method of the present invention also increases the potential utility of myocytes as vehicles for gene therapy in various ischemic events, including but not limited to myocardial infarction by more efficiently providing a large number of such cells for transfection, and also by providing a more efficient means to rapidly expand transfected myocytes. Administration of the active agents to accelerate in vivo myocyte proliferation and/or to treat myocardial injuries can be used to treat heart failure, cardiomyopathies, inflammation, infection, sepsis, ischemia, heart valve disease, myocarditis, inflammation; or myocardial ischemia and infarction; and for improvement of cardiac output by increasing stroke volume.

**Examples**

*Example 1. Myocyte Proliferation*

Monolayer cultures of 1-2 day old neonatal Sprague-Dawley rat myocytes were prepared. Minced ventricular myocardium was placed into a $Ca^{2+}$- and $Mg^{2+}$-free Hanks' salt solution buffered with 30 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4. The cells were dissociated and incubated at 37°C with a mixture of partially purified trypsin (2.4 IU/ml, Worthington Biochemcial), α-chymotrypsin (2.7 IU/ml) and elastase (0.94 IU/ml, Sigma Chemical, St. Louis, MO). After each of 5 successive 20 minute incubations, the dissociated cells were mixed with Eagle's minimal essential medium (MEM; Gibco, MD) containing 10% newborn calf serum, and were centrifuged and pooled. The dissociated cells were enriched for cardiomyocytes by the technique of differential adhesion to tissue culture plastics for 90 minutes at 37°C in a humidified 5% $CO_2$ and air atmosphere, and plated onto laminin-coated (20 μg/ml) silicone dishes at a concentration of approximately $4 \times 10^5$ cells/dish. Cultures were incubated in a humidified 5% $CO_2$, 95% air atmosphere at 37°C. After an overnight incubation in MEM containing 10% newborn calf serum and 0.1 mM 5-bromo 2 deoxyuridine (Sigma), the attached cells were rinsed in serum-free medium. Briefly, standard MEM was supplemented with MEM amino acids, vitamins, penicillin-streptomycin (GIBCO), and 2 mM glutamine. In addition, the medium contained 30 nM $NaSeO_4$, 2.5 μg/ml human insulin, 10 μg/ml human transferrin (Sigma), 0.25 mM ascorbic acid (Sigma) and 0.1 mM 5-bromo-2'-deoxyuridine to minimize the proliferation of non-myogenic cells. The medium was replaced every 2 days with fresh medium over the course of the experiments.

The results of these experiments (Table I) demonstrate that the active agents of the invention increased myocyte proliferation over that of culture medium alone.

% Increase in Cell Number

| Treatment (μg/ml) | Day 3 | Day 8 |
|---|---|---|
| No peptide | 116.7 | 133.0 |
| AII(1-7) 10 μg/ml | 183.0 | 175.0 |
| AII(1-7) 100 μg/ml | 150.0 | 191.0 |
| AII 10 μg/ml | 166.0 | 170.0 |
| AII 100 μg/ml | 200.0 | 210.0 |

*Example 2. Myocardial Repair*

Rabbits underwent a surgical procedure under intramuscular anesthesia (Ketamine Rompum) after shaving with animal clippers and preparation with betadine and isopropyl alcohol. After induction of anesthesia, the rabbits were intubated and placed on a ventilator to assist respiration. Vital signs were monitored with a pulse oximeter. A midline sternotomy was then performed. After exposure of the pericardial sac, a 3 cm incision was made into the pericardium. After visualization of the epicardial surface, two coronary arteries, the left circumflex, and the left anterior descending artery were exposed and ligated by 4-0 Vicryl suture. Vehicle (10% Hydron, 60% ethanol, and 1% polyethylene glycol), with and without peptide (1 mg/ml, 0.05 ml) was injected in the cardiac muscle distal to the site of coronary occlusion. The sternum was then closed with 2-0 silk. The muscle and skin were then closed with 3-0 Dexon II suture. Twenty-eight days after surgery, the animals were euthanized and a necropsy was performed. The number of microscopic fields with fibrosis (scar) and the number of blood vessels/field present in the infarct site were assessed by microscopic evaluation. The presence of a blood vessel was defined as a channel lined with endothelial cells that contained red blood cells (indicating that the vessels had a blood source).

AII (SEQ ID NO:1), AII(1-7) (SEQ ID NO:4), 2 GD (SEQ ID NO:39), and 9GD (SEQ ID NO:41) all decreased the size of the infarct (as measured by the number of microscopic fields with scar) (Table 2), although the decrease associated with administration of 9GD was not statistically significant, possibly due to the smaller group size. The decreases with the other peptides ranged from 30% to 85% of the infarct size found at placebo-treated sites. In contrast with that observed on day 7, the vascularization at the infarct site was not changed at this latter time point (Table 3).

Table 2. Effect of Angiotensin Peptides on the Size of Myocardial Fibrosis after Infarct Repair

| Treatment | # of animals | Mean +/- SEM | P Value |
|---|---|---|---|
| Hydron | 12 | 17.83 +/- 2.66 | |
| 2GD | 4 | 6.00 +/- 2.94 | 0.032 |
| 9GD | 2 | 11.50 +/- 6.5 | >0.05 |
| AII | 3 | 5.67 +/- 0.88 | 0.045 |
| AII(107) | 4 | 3.20 +/- 1.28 | 0.004 |

Table 3. Effect of Angiotensin Peptides on the Formation of Collateral Circulation in Ischemic Myocardial Tissue

| Treatment | # of animals | Mean +/- SEM | P Value |
|---|---|---|---|
| Hydron | 12 | 52.6 +/- 14.6 | |
| 2GD | 4 | 8.8 +/- 3.53 | 0.110 |
| 9GD | 2 | 15.95 +/- 0.95 | 0.337 |
| AII | 3 | 41.9 +/- 13.3 | 0.729 |
| AII(107) | 4 | 82.0 +/- 22.2 | 0.287 |

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.

We claim:

1. A method for promoting myocyte proliferation or differentiation, comprising contacting myocytes with an amount effective to promote myocyte proliferation or differentiation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups $R^1$-$R^8$ in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which $R^1$ and $R^2$ together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group, or is absent,

$R^A$ is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, $Me^2$Gly, Pro, Bet, Glu($NH_2$), Gly, Asp($NH_2$) and Suc,

$R^B$ is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, Tyr($PO_3$)$_2$, Thr, Ala, Ser, homoSer and azaTyr;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including $R^4$ as a terminal Tyr group, or is absent.

2. The method of claim 1 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50.

3. A method for promoting myocyte proliferation or differentiation, comprising contacting myocytes with an amount effective to promote myocyte proliferation or differentiation of at least one active agent comprising a sequence selected from the group consisting of the general formula:

Asp-Arg-R1-Tyr-Ile-His-Pro-R2

wherein R1 is selected from the group consisting of Val, Pro, Ile, norLeu, Ile, Lys, Ala, Gly, Aib, Acpc and Tyr; and

wherein R2 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, or is absent.

4. The method of claim 3 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:37, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:47.

5. The method of any one of claims 1-4 further comprising contacting the myocytes with an amount effective to promote myocyte proliferation or differentiation of at least one compound selected from the group consisting of epidermal growth factor, insulin-like growth factor, fibroblast growth factor, platelet derived growth factor, nerve growth factor, tumor necrosis factor, and interleukin I.

6. A method for promoting myocardial tissue repair following a myocardial injury, comprising administering to a patient in need thereof an amount effective to promote myocardial tissue repair following a myocardial injury of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups $R^1$-$R^8$ in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which $R^1$ and $R^2$ together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group, or is absent,

$R^A$ is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, $Me^2$Gly, Pro, Bet, Glu($NH_2$), Gly, Asp($NH_2$) and Suc,

$R^B$ is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ala, Ser, homoSer and azaTyr;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including $R^4$ as a terminal Tyr group, or is absent.

7. The method of claim 6 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50.

8. A method for promoting myocardial tissue repair following a myocardial injury, comprising administering to a patient in need thereof an amount effective to promote myocardial tissue repair following a myocardial injury of at least one active agent comprising a sequence selected from the group consisting of the general formula:

Asp-Arg-R1-Tyr-Ile-His-Pro-R2

wherein R1 is selected from the group consisting of Val, Pro, Ile, norLeu, Ile, Lys, Ala, Gly, Aib, Acpc and Tyr; and

wherein R2 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, or is absent.

9. The method of claim 8 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:37, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:47.

10. The method of any one of claims 6-9 further comprising administering an amount effective to promote myocardial tissue repair following a myocardial injury of at least one active agent selected from the group consisting of free radical scavengers, calcium

antagonists, β-blockers, magnesium, inhibitors of white blood cell function, inhibitors of cellular adhesion selectin molecules, adenosine, fibroblast growth factor, digoxin, and ACE inhibitors.

11. A kit for promoting myocyte proliferation or differentiation, comprising

(a) an amount effective to promote myocyte proliferation or differentiation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups $R^1$-$R^8$ in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which $R^1$ and $R^2$ together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group, or is absent,

$R^A$ is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, $Me^2$Gly, Pro, Bet, Glu($NH_2$), Gly, Asp($NH_2$) and Suc,

$R^B$ is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, Tyr($PO_3$)$_2$, Thr, Ala, Ser, homoSer and azaTyr;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including $R^4$ as a terminal Tyr group, or is absent; and

(b) instructions for using the active agent to promote myocyte proliferation or differentiation.

12. The kit of claim 11 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29,

SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50.

13. A kit for promoting myocyte proliferation or differentiation, comprising

(a) an amount effective to promote myocyte proliferation or differentiation of at least one active agent comprising a sequence selected from the group consisting of the general formula:

Asp-Arg-R1-Tyr-Ile-His-Pro-R2

wherein R1 is selected from the group consisting of Val, Pro, Ile, norLeu, Ile, Lys, Ala, Gly, Aib, Acpc and Tyr;

wherein R2 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, or is absent; and

(b) instructions for using the active agent to promote myocyte proliferation or differentiation.

14. The kit of claim 13 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:37, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:47.

15. The kit of any one of claims 11-14 further comprising contacting the myocytes with an amount effective to promote myocyte proliferation or differentiation of at least one compound selected from the group consisting of epidermal growth factor, insulin-like growth factor, fibroblast growth factor, platelet derived growth factor, nerve growth factor, tumor necrosis factor, and interleukin I.

16. A pharmaceutical composition comprising:

(a) an amount effective to promote myocyte proliferation or differentiation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups $R^1$-$R^8$ in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which $R^1$ and $R^2$ together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group, or is absent,

$R^A$ is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, $Me^2Gly$, Pro, Bet, Glu($NH_2$), Gly, Asp($NH_2$) and Suc,

$R^B$ is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, Tyr($PO_3$)$_2$, Thr, Ala, Ser, homoSer and azaTyr;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including $R^4$ as a terminal Tyr group, or is absent; and

(b) a pharmaceutically acceptable carrier.

17. The pharmaceutical composition of claim 16 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50.

18. The pharmaceutical composition of claim 16 further comprising an amount effective to promote myocyte proliferation and differentiation of a compound selected from the group consisting of epidermal growth factor, insulin-like growth factor, fibroblast growth factor, platelet derived growth factor, nerve growth factor, tumor necrosis factor, and interleukin I.

19. A pharmaceutical composition comprising:

(a) an amount effective to promote myocyte proliferation or differentiation of at least one active agent comprising a sequence selected from the group consisting of the general formula:

Asp-Arg-R1-Tyr-Ile-His-Pro-R2

wherein R1 is selected from the group consisting of Val, Pro, Ile, norLeu, Ile, Lys, Ala, Gly, Aib, Acpc and Tyr;

wherein R2 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, or is absent; and

(b) a pharmaceutically acceptable carrier.

20. The pharmaceutical composition of claim 18 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:37, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:47.

21. The pharmaceutical composition of claim 20 further comprising an amount effective to promote myocyte proliferation and differentiation of a compound selected from the group consisting of epidermal growth factor, insulin-like growth factor, fibroblast growth factor, platelet derived growth factor, nerve growth factor, tumor necrosis factor, and interleukin I.

22. A pharmaceutical composition comprising:

(a) an amount effective to promote myocardial repair following myocardial injury of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups $R^1$-$R^8$ in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which $R^1$ and $R^2$ together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group, or is absent,

$R^A$ is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, $Me^2$Gly, Pro, Bet, Glu($NH_2$), Gly, Asp($NH_2$) and Suc,

$R^B$ is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, Tyr($PO_3$)$_2$, Thr, Ala, Ser, homoSer and azaTyr;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including $R^4$ as a terminal Tyr group, or is absent; and

(b) a pharmaceutically acceptable carrier.

23. The pharmaceutical composition of claim 20 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50.

24. The pharmaceutical composition of claim 22 further comprising an amount effective to promote myocardial repair following myocardial injury of a compound selected from the group consisting of free radical scavengers, calcium antagonists, β-blockers, magnesium, inhibitors of white blood cell function, inhibitors of cellular adhesion selectin molecules, adenosine, fibroblast growth factor, digoxin, and ACE inhibitors.

25. A pharmaceutical composition comprising:

(a) an amount effective to promote myocardial repair following myocardial injury of at least one active agent comprising a sequence selected from the group consisting of the general formula:

Asp-Arg-R1-Tyr-Ile-His-Pro-R2

wherein R1 is selected from the group consisting of Val, Pro, Ile, norLeu, Ile, Lys, Ala, Gly, Aib, Acpc and Tyr;

wherein R2 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, or is absent; and

(c) a pharmaceutically acceptable carrier.

26. The pharmaceutical composition of claim 25 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:37, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:47.

27. The pharmaceutical composition of claim 25 further comprising an amount effective to promote myocardial repair following myocardial injury of a compound selected from the group consisting of free radical scavengers, calcium antagonists, β-blockers, magnesium, inhibitors of white blood cell function, inhibitors of cellular adhesion selectin molecules, adenosine, fibroblast growth factor, digoxin, and ACE inhibitors;

28. An improved cell culture medium for promotion of myocyte proliferation or differentiation, the improvement comprising providing an amount effective to promote myocyte proliferation or differentiation of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups $R^1$-$R^8$ in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which $R^1$ and $R^2$ together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group, or is absent,

$R^A$ is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, $Me^2$Gly, Pro, Bet, Glu($NH_2$), Gly, Asp($NH_2$) and Suc,

$R^B$ is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, Tyr($PO_3$)$_2$, Thr, Ala, Ser, homoSer and azaTyr;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including $R^4$ as a terminal Tyr group, or is absent.

29. The improved cell culture medium of claim 28 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3,

SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50.

30. An improved cell culture medium for promotion of myocyte proliferation or differentiation, the improvement comprising providing an amount effective to promote myocyte proliferation or differentiation of at least one active agent comprising a sequence selected from the group consisting of the general formula:

Asp-Arg-R1-Tyr-Ile-His-Pro-R2

wherein R1 is selected from the group consisting of Val, Pro, Ile, norLeu, Ile, Lys, Ala, Gly, Aib, Acpc and Tyr;

wherein R2 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, or is absent.

31. The improved cell culture medium of claim 30 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:37, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:47.

32. A method for treating heart failure in a mammal, comprising administering an amount effective to treat heart failure of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups $R^1$-$R^8$ in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7R^8$$

in which $R^1$ and $R^2$ together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group, or is absent,

$R^A$ is suitably selected from H, Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, $Me^2$Gly, Pro, Bet, Glu($NH_2$), Gly, Asp($NH_2$) and Suc,

$R^B$ is suitably selected from Arg, Lys, Ala, Orn, Citron, Ser(Ac), Sar, D-Arg and D-Lys;

$R^3$ is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

$R^4$ is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ala, Ser, homoSer and azaTyr;

$R^5$ is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

$R^6$ is His, Arg or 6-$NH_2$-Phe;

$R^7$ is Pro or Ala; and

$R^8$ is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including $R^4$ as a terminal Tyr group, or is absent.

33. The method of claim 32 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO: 32, SEQ ID NO:33, SEQ ID NO: 34; SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50.

34. A method for treating heart failure in a mammal, comprising administering an amount effective to treat heart failure of at least one active agent comprising a sequence selected from the group consisting of the general formula:

Asp-Arg-R1-Tyr-Ile-His-Pro-R2

wherein R1 is selected from the group consisting of Val, Pro, Ile, norLeu, Ile, Lys, Ala, Gly, Aib, Acpc and Tyr; and

wherein R2 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, or is absent.

35. The method of claim 34 wherein the active agent is selected from the group consisting of angiotensinogen, SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:37, SEQ ID NO:40, SEQ ID NO:41, and SEQ ID NO:47.

SEQUENCE LISTING

<110> Rodgers, Kathleen
diZerega, Gere

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<130> 98068B

<140> To be assigned
<141> 2000-03-09

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1 5

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1 5

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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 00/53211 A3

(54) Title: METHOD OF PROMOTING MYOCYTE PROLIFERATION AND MYOCARDIAL TISSUE REPAIR

(57) Abstract: The present invention provides methods, pharmaceutical compositions, improved cell culture medium and kits for promoting myocyte proliferation and myocardial tissue repair following myocardial injury by contact with angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and/or AII $AT_2$ type 2 receptor agonists.

# INTERNATIONAL SEARCH REPORT

Intrnt tional Application No
PCT/US 00/06198

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/08 A61K38/06 A61K38/07 C12N5/08 A61P9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

STRAND, BIOSIS, EPO-Internal, PAJ, WPI Data, MEDLINE, EMBASE, LIFESCIENCES, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | SUN YAO ET AL: "Angiotensin II, transforming growth factor-betal and repair in the infarcted heart." JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY, vol. 30, no. 8, August 1998 (1998-08), pages 1559-1569, XP002150870 ISSN: 0022-2828 the whole document | 1-35 |
| A | US 5 716 935 A (RODGERS K.E. ET AL.) 10 February 1998 (1998-02-10) the whole document | 1-35 |
| A | WO 98 26795 A (THE UNIVERSITY OF SOUTH CALIFORNIA) 25 June 1998 (1998-06-25) page 42 -page 44 | 1-35 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 24 October 2000 | 07/11/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Moreau, J |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | SUN YAO ET AL: "Inhibition of angiotensin-converting enzyme and attenuation of myocardial fibrosis by lisinopril in rats receiving angiotensin II." JOURNAL OF LABORATORY AND CLINICAL MEDICINE, vol. 126, no. 1, 1995, pages 95-101, XP000953010 ISSN: 0022-2143 the whole document | 1-35 |

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Information on patent family members

Int tional Application No
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